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PRINCIPAL INVESTIGATOR: Dennis J. Slamon, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California Los Angeles
Los Angeles, California 90024-1406

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The alteration of the HER-2/*neu* gene has been shown to correlate with a poor prognosis in those patients whose tumors contain it. This has led to studies from our laboratory indicate that it may play a role in the pathogenesis of the disease for some patients. Given that the HER-2/*neu* gene encodes a growth factor receptor found on the membrane of tumor cells and given its potential role in the pathogenesis of some human breast cancers, it is a logical target for the development of new therapeutic approaches directed at this alteration. Studies with monoclonal antibodies directed against the extra cellular domain of the receptor indicate that many may have significant growth inhibitory properties. Recently ligands have been identified which interact either directly or indirectly with the human HER-2/*neu* receptor, however little is known about the biologic effects of these molecules. There is some controversy as to whether the ligands mediate growth stimulatory or growth inhibitory effects or both. A greater understanding about the biologic effects of HER-2/*neu* overexpression as well as the impact of agonists and antagonists to the receptor will be required to fully therapeutically exploit this gene alteration in human breast cancer. Finally, little is know about the biologic effects of other molecular alterations which may occur in combination with HER-2/*neu* expression.

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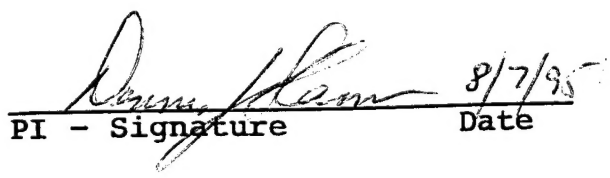

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PROGRESS REPORT

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BIOLOGIC EFFECTS OF HER-2/*neu* GENE OVEREXPRESSION AND AGONISTS AND ANTAGONISTS TO THE RECEPTOR IN HUMAN BREAST CANCER

PRINCIPAL INVESTIGATOR: Dennis J. Slamon, M.D., Ph.D.

The background and introduction to this Progress Report remain the same as in the initial proposal and are restated here.

INTRODUCTION

The HER-2/*neu* gene is a member of the proto-oncogene family. It was initially identified in studies in which pregnant female rats were exposed to a chemical carcinogen resulting in the development of a tumor, neuroglioblastoma, in their offspring , (9). DNA extracted from these tumors is capable of transforming NIH 3T3 cells, indicating the presence of a dominant transforming oncogene. When the gene responsible for this transforming activity was cloned and characterized, it was found to be analogous to but distinct from the proto-oncogene encoding the epidermal growth factor receptor i.e. the c-*erb* B gene, (10-12). The human homologue for this dominant oncogene was cloned and characterized, and termed HER-2/*neu* or c-*erb*-B2 (10-12). For the past nine years our laboratory has had an NIH-funded screening program designed to test DNA, RNA and protein from human tumor tissues to determine if alterations in structure or expression exist in proto-oncogenes, growth factor, growth factor receptors, or tumor suppressor genes. These studies were undertaken to both identify common alterations in specific tumor types as well as to determine whether or not the alterations correlate with any clinical parameters of the tumors in which they are found. As part of this screening program we used the HER-2/*neu* gene as a probe, while looking for alterations in a large number of different human malignancies. This screening procedure demonstrated amplification of the gene in approximately 25% of human breast cancers (13, 14). When the alteration was examined for association with clinical parameters, it was found that gene amplification was associated with a poor prognosis; i.e. those women whose tumor contained the alteration had a shorter disease-free and overall survival (13,14). This association was initially disputed (15-21), but a large number of subsequent studies have now confirmed it (22-44). Moreover, the prognostic association between HER-2/*neu* alteration and disease outcome is seen in both node positive and node negative breast cancer. This literature and the controversy surrounding the association has been extensively reviewed recently (see Ref. 43 discussion section). The bulk of the published data now clearly support the prognostic significance of HER/*neu* overexpression in breast cancer (43). However, all of the reviewed studies were retrospective analyses of archival cohorts of specimens. Most recently the first prospective study, consisting of 1056 primary human breast cancers, was completed and published and this study also confirmed the prognostic significance of HER-2/*neu* alteration in both node negative and node positive disease (44). Taken together, these data confirm the prognostic significance of HER-2/*neu* amplification/overexpression in human breast cancer.

There are at least two possible explanations for the association between HER-2/*neu* amplification/overexpression and poor clinical outcome; a) the alteration serves as a genetic marker for a poor outcome but plays no role in causing it; i.e. a useful prognostic epiphenomenon; or alternatively b) the alteration is associated with a poor outcome because it plays a direct role in the pathogenesis of such an outcome. There is circumstantial evidence which supports the latter possibility. These data include the following: 1) the mutated rat *neu* gene can act as a dominant transforming oncogene, underscoring its oncogenic potential and NIH 3T3 cells transfected with the gene are not only transformed *in vitro*, but are tumorigenic in the nude mouse (9, 10, 45, 46); 2) monoclonal antibodies directed against the extracellular domain of the rat *neu* gene will inhibit transformation *in vitro* as well as tumorigenicity *in vivo* (46, 47); 3) studies to develop transgenic mice using the mutated rat gene under the control of an MMTV promoter, demonstrate that these mice develop breast cancer at three months of age (48); and finally, 5) studies using a transfected human HER-2/*neu* gene in NIH 3T3 cells demonstrate that it too will transform the cells and that higher levels of expression result in greater transforming efficiency, and greater tumorigenicity of the cells (49, 50). In composite, these data clearly show the ability of the HER-2/*neu* gene to mediate transformation *in vitro* and tumorigenicity *in vivo*.

Each of these lines of circumstantial evidence, however, have shortcomings with regards to implicating the HER-2/*neu* gene in the pathogenesis of human breast cancer. The first line of evidence proved that the rat *neu* gene could be oncogenic; however, sequence analysis showed the gene to have a point mutation in the transmembrane domain (45). To date, no such mutation has been identified in the human HER-2/*neu* gene (14, 51). Instead the alteration found in human breast cancer is amplification and overexpression of the normal gene. In addition, the mutated rat *neu* gene induces neuroglioblastomas and not breast cancer in the animals (9). The monoclonal antibody studies were equally convincing that the mutated rat gene could have a role in transformation of neural tissue but, again, these antisera were not directed against the human protein nor were they used in altering the phenotype of human breast carcinoma cells overexpressing a normal, non-mutated, human HER-2/*neu* gene. The transgenic mouse studies were particularly compelling in showing that alterations in the rat *neu* gene could result in the development of breast carcinoma but, again, the study had used the mutated rat gene (48). Lastly, the data demonstrating that an overexpressed, <u>non-mutated</u> human HER-2/*neu* gene could transform NIH 3T3 cells proved that the human gene was oncogenic *in vitro* (49, 50), but the experiments again used NIH 3T3 cells, not human breast cancer or breast epithelial cells. In addition, the levels of overexpression in these studies were far in excess of what is seen in most human breast cancer specimens in nature (14, 51). To circumvent some of the concerns raised with the experiments utilizing the rat *neu* gene or murine cells (NIH-3T3), we recently designed a series of experiments to introduce the human gene into human breast cancer cells as well as non-transformed immortalized and normal non-immortalized human breast epithelial cells. Important in the studies is to mimic as closely as possible the alteration seen in human tumors and then determine the biologic effects (if any) of this alteration. Central to these studies was the use of the human gene in human cells and the objective to hold the levels of overexpression at or below those seen in actual human tumors; i.e. not to exceed levels found in primary and/or metastatic tumors in nature. These studies have not yet been published and have just recently been completed. They are presented as preliminary data at the beginning of the Methods Section, since the techniques used and assays developed for their completion are central to the proposed studies. We feel that they demonstrate our ability to conduct the studies detailed in this application.

In addition we wish to examine the effects that an additional alteration, mutation of the p53 gene, may have on cells overexpressing the HER-2/*neu* gene. There is considerable evidence that the conversion of a normal cell into a malignant cell is a multistep process which may involve the alteration of more than one if not several genes (52). Mutation of the p53 gene is one of the most common genetic alterations found in human malignancies (53) and is frequently found in breast cancer (53-71). The mutation rate in breast cancer is reported to be from a low of 14% (65) to a high of 58% (70) with the most frequently reported rate being between 25-30% of all cases. This incidence makes p53 alteration a potentially important mutation in the pathogenesis of human breast cancer. Similar to the data with HER-2/*neu*, this concept is circumstantially supported by data indicating that p53 mutation is associated with aggressive subtypes of the disease and/or a poor prognosis (56, 58, 64-66, 68, 70, 71). The concept gains further support with experimental data demonstrating that introduction of a wild type p53 gene into a human breast cancer cell line containing a mutant gene will suppress the transformed phenotype (72). Data are now accumulating which indicate that alterations in the p53 gene are frequently associated with HER-2/*neu* alterations (59,61,65). This combination of mutations may be very important in the pathogenesis of some human breast cancers. The HER-2/*neu* overexpressing cell lines already developed (see below) as well as those proposed to be developed as part of this application should be useful in addressing this issue.

BODY OF REPORT

The experimental methods used for the studies proposed in this program are unchanged from those detailed in the initial proposal and will be described in brief in this Program Report on the basis of a Specific Aim by Specific Aim approach.

Specific Aim I - To further develop a series of human breast epithelial and cancer cell lines containing defined alterations in expression of the human HER-2/*neu* gene.

This specific aim has largely been accomplished in the first nine months of funding for this project (11/94 - 7/95). The methods used to achieve the goals of this Specific Aim involve the introduction of a full length human HER-2/*neu* c-DNA into a series of human breast epithelial cell lines representing normal breast epithelial cells, immortalized but non-transformed breast epithelial cells and breast cancer cells. The cell lines detailed in the proposal, i.e. T47D, MDA-MB-231, MDA-MB-435, BT-20 and BT-483 have all been successfully transfected and engineered to overexpress the HER-2/*neu* gene. These transfectants have been characterized for stable HER-2/*neu* overexpression and all appear to have this feature (at least at 6-months of follow-up). The biologic characterization of these cells has been similar to those studies presented in the preliminary data in the initial proposal, i.e. ^{3}H-thymidene incorporation, cell growth (*in vitro)* anchorage independent growth and tumorigenicity. In all assays the data for the newly established engineered cells are similar to the data for the MCF-7, B5 and HBL-100 cell lines. DNA synthetic rate increases significantly as does cell growth, anchorage independent growth and tumorigenicity. These data are important in that they demonstrate that the biologic effects of HER-2/*neu* overexpression seen in the MCF-7, B5 and HBL-100 cell lines are not restricted to just those cell lines but can also be achieved in all of the breast cancer cell lines evaluated. These findings lend substantial credence to the concept that overexpression of the HER-2/*neu* gene plays an important pathogenic role in the aggressive biologic behavior of those cells and tumors which contain it. More recent studies using some of these cell lines has lead to insights into the clinical observation that human breast cancers which overexpress

the HER-2/*neu* receptor tend to be estrogen receptor negative. Studies performed in our laboratory and, in part, supported by the grant, have demonstrated a potentially important direct interaction between activation of the HER-2/*neu* receptor and down regulation of the estrogen receptor. These data have recently been published and a reprint of the work is included in the appendix section of this report.

Specific Aim II - To assess the biologic effects of agonists, i.e. the heregulin and *neu* differentiation factor ligands as well as an antagonist, i.e. a monoclonal antibody, to the HER-2/*neu* reception on human breast cells, *in vitro.*

The objective of this Specific Aim is to assess the biologic effects of HER-2/*neu* agonists and antagonists *in vitro.* The methods for this aim are detailed in the initial proposal and are unchanged. They will be mentioned in brief. The *in vitro* assays ^{3}H-thymidene incorporation, cell proliferation assays, clonogenic assays on plastic, anchorage independent growth assays (soft agar colony formation) and differentiation studies as determined by analysis of differentiation marker expression. In the past nine months we have completed the ^{3}H-thymidene assay, cell proliferation assays, clonogenic assays and soft agar assays for the MCF-7, SKBr3, MDA-MB-231, MDA-MB-435, and BT-20 cells using the heregulin agonist and the 4D5 antagonist. These data demonstrate that heregulin uniformly induces proliferation in all of these assays to a greater or lesser degree. In addition, those cells expressing higher levels of HER-2/*neu* had a greater growth stimulating response to heregulin. There was absolutely no evidence of growth inhibition as measured by any of the assays in any of the cell lines treated with heregulin. These data provide convincing evidence that heregulin is a growth stimulatory ligand *in vitro.* The studies of heregulin effects on differentiation have not yet been initiated and will begin in the next year of funding. The studies involving the effects of NDF in these systems have also not yet begun and will begin in the coming year.

Specific Aim III - To assess the biologic effects of the ligands and antibody alone and in combination on HER-2/*neu* expressing human breast cancer cells growing *in vivo.*

In the first nine months of funding we have been able to successfully conduct preliminary studies on the tumorigenicity of HER-2/*neu* transfected MCF-7 cells in response to heregulin and the 4D5 monoclonal antibody. These studies demonstrate that heregulin again has a growth stimulatory effect with enhancement of both tumor growth rate and size *in vivo.* The 4D5 studies presented in the preliminary data of the initial proposal have been repeated and confirmed in the past nine months and clearly demonstrate the growth inhibitory effects of the monoclonal antibody. The metastatic assays proposed in the Specific Aim have recently been initiated but have not yet been completed. We anticipate preliminary data on this portion of the Specific Aim in the upcoming funding year.

Specific Aim IV - To assess the biologic effects of an additional molecular alteration i.e. mutation of the p53 gene in combination with HER-2/*neu* overexpression in human breast cells both *in vitro* and *in vivo*

None of the studies proposed in this Specific Aim have been undertaken in the first nine months of funding. We hope to begin these studies in the upcoming funding year after completion of a number of studies listed in the first three Specific Aims.

CONCLUSIONS

Based on the data obtained during the first nine months of funding for this proposal (11/84-7/95) we have developed data addressing several potentially important aspects of the HER-2/*neu* alteration in human breast cancer. These include the following:

A.. Introduction of HER-2/*neu* overexpression into human breast cancer cells which do not contain it will lead to significant changes in the biologic behavior of these cells both *in vitro* and *in vivo*. These effects include an increase in DNA synthetic rate, an increase in growth rate *in vitro*, and an increase in tumorigenicity. These preliminary data in a wide variety of human breast cancer cell lines are consistent with the concept that this alteration is an important pathogenic alteration in those human breast cancers in which it occurs accounting, at least in part, for the adverse prognostic significance of its presence.

B. Antagonists to the HER-2/*neu* receptor such as the 4D5 monoclonal antibody clearly inhibit the *in vitro* growth of cells which overexpress the receptor. Conversely the heregulin ligand will stimulate growth of those same cells. These data lead to the possible development of therapeutic strategies directed at exploiting the pathogenic role of the alteration.

C. The data on the *in vivo* effects of heregulin and the 4D5 monoclonal antibody are consistent with the *in vitro* data in that heregulin clearly reduces increased tumor growth while the antibody consistently inhibits tumor growth. Again these data provide potential therapeutic directions for use of antagonists to HER-2/*neu* in those patients whose tumors contain HER-2/*neu* overexpression.

In summary, the data generated to date clearly indicate a pathogenic role for HER-2/*neu* overexpression in some human breast cancers (i.e. the 30% in which it occurs). The data from studies using agonists and antagonists to this receptor can provide significant information for understanding the basic biology of human breast cancer cells as well as potential new therapeutic approaches to the disease.

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HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells

Richard J Pietras[1], Jane Arboleda[1], David M Reese[1], Nancy Wongvipat[1], Mark D Pegram[1], Lillian Ramos[1], Cornelia M Gorman[2,4], Malcolm G Parker[3], Mark X Sliwkowski[2] and Dennis J Slamon[1]

[1]*UCLA School of Medicine, Department of Medicine, Division of Hematology-Oncology, Los Angeles, CA 90095, USA;* [2]*Genentech, 450 Point San Bruno Blvd., San Francisco, CA 94080, USA;* [3]*Imperial Cancer Research Fund, Lincoln's Inn Fields, London, England, UK;* [4]*Megabios Corporation, 863A Mitten Road, Burlingame, CA 94010*

Growth of human breast cells is closely regulated by steroid hormone as well as peptide hormone receptors. Members of both receptor classes are important prognostic factors in human breast cancer. Clinical data indicate that overexpression of the HER-2 gene is associated with an estrogen receptor-negative phenotype. In this study, we demonstrate that introduction of a HER-2 cDNA, converting non-overexpressing breast cancer cells to those which overexpress this receptor, results in development of estrogen-independent growth which is insensitive to both estrogen and the antiestrogen, tamoxifen. Moreover, activation of the HER-2 receptor in breast cancer cells by the peptide growth factor, heregulin, leads to direct and rapid phosphorylation of ER on tyrosine residues. This is followed by interaction between ER and the estrogen-response elements in the nucleus and production of an estrogen-induced protein, progesterone receptor. In addition, overexpression of HER-2 receptor in estrogen-dependent tumor cells promotes ligand-independent down-regulation of ER and a delayed autoregulatory suppression of ER transcripts. These data demonstrate a direct link between these two receptor pathways and suggest one mechanism for development of endocrine resistance in human breast cancers.

Keywords: HER-2/neu ; estrogen receptor; heregulin; tyrosine phosphorylation; breast cancer

Introduction

Estrogens and peptide growth factors control the proliferation of breast cells. Alterations in the receptors for these agonists occur in human cancers in nature and lead to disruption of growth regulation (Harris et al.,1992). Among growth factor receptors, the most frequently implicated in human cancers have been members of the class I receptor tyrosine kinase family (erb B). Erb B tyrosine kinase receptors are overexpressed in two-thirds of human breast cancers and are associated with malignant transformation (Slamon et al.,1987; Slamon et al., 1989; Harris et al.,1992; Dougall et al.,1994). These receptors include the HER-2 (erb B2) and HER-3 (erb B3) proteins which, together, constitute a high affinity functional receptor for heregulin (HRG), a ligand implicated in the autocrine/paracrine growth of breast epithelial cells (Carraway & Cantley,1994; Sliwkowski et al.,1994). Receptors for estrogen are part of a family of steroid hormone receptors related to the viral erb A gene (Green & Chambon, 1988), and like the erb B proteins these receptors may play important pathogenic roles in breast cancer. Cross-coupling between erb B and estrogen receptor (ER) signal pathways in rodent uterine tissues has been reported (Ignar-Trowbridge et al.,1992) and is reminiscent of the cooperativity between viral erb A and erb B oncogenes in the malignant transformation of avian hematopoietic cells (Beug & Graf,1989). Direct interaction between erb B signal pathways and ER in human breast cancer cells is the subject of the current studies.

Upon estradiol binding, ER interacts with specific estrogen-response elements (ERE) in the vicinity of target genes and modulates their transcription (Green & Chambon,1988; Smith et al.,1993). The HER-2 receptor, with intrinsic tyrosine kinase activity, is believed to promote signal transduction along specific phosphorylation cascades (Harris et al.,1992; Silvennoinen et al.,1993; Dougall et al.,1994), with recruitment of proteins that serve as a link in activation of ras, inositol triphosphate, and, possibly, other signaling pathways to the nucleus (Silvennoinen et al.,1993). Phosphorylation of ER on tyrosine and/or serine residues has been associated with functional changes in both hormone binding and nuclear localization (Arnold et al.,1994; Kuiper & Brinkmann,1994; Le Goff et al.,1994) and may be a link to kinase-mediated growth factor pathways. Blockade of estrogen-induced growth of breast tumor cells by tyrosine kinase inhibitors provides further evidence of the importance of tyrosine kinase pathways in estrogen action (Reddy et al.,1992).

Expression of either HER-2 or ER in human breast cancer provides important prognostic information (Slamon et al.,1987; Slamon et al.,1989b; Nicholson et al.,1990; Benz et al.,1992; Wright et al.,1992; Borg et al.,1994; Elledge et al.,1994). There are considerable data showing an association between HER-2 overexpression and the ER-negative phenotype (Zeillinger et al.,1989; Adnane et al.,1989), and failure of antiestrogen therapy in patients with breast cancer correlates with erb B receptor expression (Nicholson et al.,1990; Wright et al.,1992). In view of the above data, a greater understanding of the possible influence of erb B genes on the estrogen response is needed. Although ER is known to modulate HER-2 gene expression (Read et al.,1990; Russell & Hung,1992), we postulate that reciprocal regulation of ER by erb B pathways may also

Correspondence: Richard J Pietras
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et al., 1989; Zeillinger et al., 1989), we investigated the potential effect of HER-2 overexpression on ER expression and the genesis of estrogen-independent growth. This was accomplished using MCF-7 breast cancer cells which have single copies of HER-2 gene, no expression of HRG and require estrogen for growth (Read et al., 1990; Holmes et al.,1992). Transfection of a full length HER-2 cDNA into MCF-7 cells using a retroviral vector results in the introduction of 2-5 copies of the gene /cell and overexpression of HER-2 receptor (MCF-7 HER-2; Chazin et al., 1992). As shown in Fig. 1A, estradiol promotes a dose-dependent increase in proliferation of MCF-7 control cells in vitro ($P<0.001$), but hormone doses of up to 5 nM elicit no significant effect on growth of MCF-7 HER-2 cells. As expected, treatment of MCF-7 parental cells with tamoxifen results in a significant reduction in cell proliferation ($P<0.01$; Fig.1B). However, MCF-7 HER-2 cells are unaffected by tamoxifen. These data indicate that overexpression of HER-2 gene in MCF-7 cells promotes insensitivity to both estradiol and tamoxifen in vitro.

Antiestrogen sensitivity of MCF-7 breast cancer cells was also tested in vivo using ovariectomized, athymic mice primed with estrogen for 10 days. Confirming prior studies (cf. Vignon et al., 1987; Wakeling, 1993), growth of MCF-7 control cells is markedly inhibited by tamoxifen (Fig.1C) while MCF-7 HER-2 cells derived from these control cells exhibit resistance to tamoxifen treatment (Fig.1D). Thus, overexpression of HER-2 receptors in human breast cancers growing in vivo is associated with failure of tamoxifen therapy and is consistent with what is seen clinically, i.e. HER-2-overexpressing tumors are resistant to tamoxifen.

To further confirm a link between HER-2 expression and hormone response, we tested the effects of an antibody to HER-2 on hormone response. If HER-2 overexpression plays a direct role in hormone resistance, then down-regulation of HER-2 receptor may result in a reversion to a more hormone-responsive phenotype. Monoclonal antibody rhuMAb HER-2 is a humanized form of the murine 4D5 antibody which is directed to the external domain of HER-2 and inhibits growth of cells with HER-2 overexpression (Carter et al., 1992). The antibody, a partial or weak agonist to the HER-2 receptor, promotes its down-regulation and blocks cell proliferation (Shepard et al., 1991). MCF-7 HER-2 cells were used to evaluate the effect of the antibody on the response of HER-2-overexpressing cells to tamoxifen therapy in vitro. As expected, rhuMAb HER-2 reduces breast cell proliferation to $88\pm3\%$ of controls when given alone (data not shown), but, more importantly, a further suppression of cell growth occurs with treatment with the antireceptor antibody and tamoxifen, indicating a return to hormone responsiveness (Fig.1B). The latter effect is not significantly different from the response to tamoxifen alone in MCF-7 parent cells (Fig.1B). These studies further support the involvement of the HER-2 receptor in mediation of hormone sensitivity.

Heregulin Activates the HER-2 Receptor and Stimulates Breast Cancer Cell Growth in the Absence of Estrogen

Heregulin-ß1 is a recombinant peptide with an EGF-like domain (Holmes et al., 1992) which activates the HER-2 receptor protein, a transmembrane tyrosine kinase. The recombinant HRG exhibits high-affinity binding to HER-2 / HER-3 heterodimers resulting in phosphorylation of HER-2 and enhanced proliferation of breast tumor cells in vitro (Holmes et al., 1992; Sliwkowski et al., 1994). To confirm

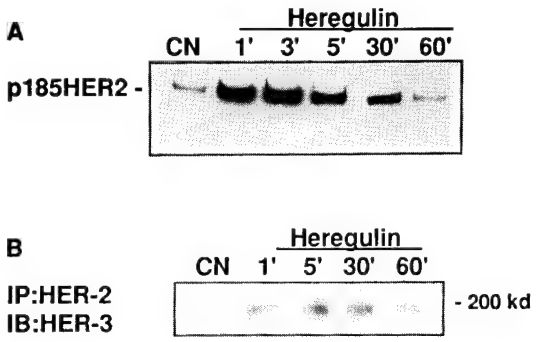

Figure 2 Heregulin promotes time-dependent HER-2 receptor phosphorylation and enhances HER-2 and HER-3 receptor association in MCF-7 HER-2 cells. (**A**) Effect of HRG treatment on HER-2 receptor phosphorylation. MCF-7 HER-2 cells were treated in vitro with HRG at 10 nM for 1 to 60 minutes. Lysates were prepared and processed as described in Materials and Methods. Samples were immunoprecipitated with anti-phosphotyrosine antibody before electrophoresis and Western blotting with anti-HER-2 antibody. HER-2 normally occurs as a 185 kd protein (Slamon et al., 1987). (**B**) Effect of heregulin on association of HER-2 and HER-3 receptors. MCF-7 HER-2 cells were treated in vitro with HRG at 10 nM for 1 to 60 minutes. Lysates were prepared and processed as above. Samples were immunoprecipitated with anti-HER-2 antibody (IP:HER-2; Slamon et al.,1987; Slamon et al., 1989a) prior to electrophoresis and Western blotting with anti-HER-3 antibody (IB:HER-3). HER-3 normally occurs as a 180 kd protein (Sliwkowski et al., 1994)

the activation of HER-2 with heregulin treatment in our system, we assessed in vitro tyrosine phosphorylation of the MCF-7 HER-2 cells in response to HRG. After HRG administration, these cells show a marked time-dependent increase in HER-2 tyrosine phosphorylation which becomes evident after one minute and peaks within thirty minutes (Fig. 2A). In addition, treatment with HRG in MCF-7 HER-2 cells promotes the enhanced association of HER-2 and HER-3 receptors, which becomes evident within one minute after HRG stimulation (Fig. 2B). These observations are consistent with earlier reports showing that HER-2 / HER-3 receptor heterodimers comprise a high-affinity receptor for HRG (Sliwkowski et al., 1994).

Due to its activating effect on the HER-2 kinase, activity of HRG on hormone-dependent growth of MCF-7 breast cells was evaluated (Fig. 3A). MCF-7 cells were implanted in ovariectomized mice without estrogen and treated with HRG or estradiol for 3 wks. Estrogen-dependent MCF-7 parent cells fail to grow in ovariectomized mice in the absence of estrogen, and, as expected, estradiol promotes an increase in growth of MCF-7 tumor nodules ($P<0.001$; Fig.3A). HRG treatment can also maintain the growth of these cells in ovariectomized mice even in the absence of estrogen ($P< 0.001$). These results suggest either that HRG stimulates an alternate growth pathway in the absence of hormone or that HRG itself may be an estrogen-related growth factor that directly acts in estrogen-dependent growth of breast cancer.

To further assess the effects of HRG on hormone-dependent growth, MCF-7 cells were tested after stable transfection with a full-length HRG-ß1 cDNA (Holmes et al., 1992). MCF-7 control cells do not produce HRG, but the transfected MCF-7 cells, designated MCF-7 HRG, show substantial levels of HRG expression, with cellular

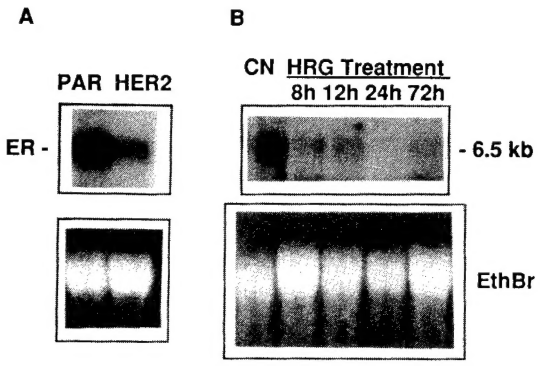

Figure 5 Activation or overexpression of HER-2 receptor elicits down-regulation of estrogen receptor transcripts. (**A**) Analyses of RNA from MCF-7 control (PAR) and MCF-7 HER-2 cells. Cell RNA was isolated 7 days after plating cells in estrogen-free media and subjected to Northern blot analysis (Chazin et al., 1992; Slamon et al., 1987), with hybridization of the resulting blot with human ER cDNA. A major ER mRNA transcript of approximately 6.5 kilobases has been described before. The ethidium bromide staining pattern of matched ribosomal RNA is shown for comparison. (**B**) Heregulin administration in vivo down-regulates ER transcripts in MCF-7 HER-2 cells grown as xenografts in athymic, ovariectomized mice over 72 hours. At the time of cell inoculation sc ($5x10^7$ cells/mouse), treatment with HRG at 2 mg/kg was started sc and continued every other day. Paired mice were given vehicle control (CON) as shown at time zero for comparison. The ethidium bromide staining pattern of matched ribosomal RNA is shown for comparison

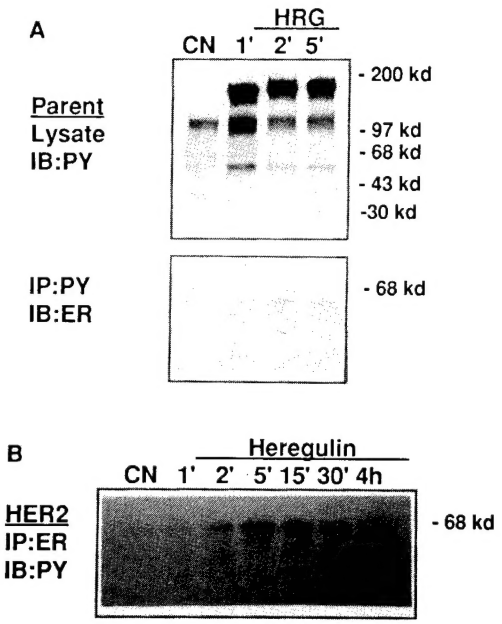

Figure 6 Activation of HER-2 receptor leads to tyrosine phosphorylation of estrogen receptor. (**A**) MCF-7 parent cells were treated in vitro with 10 nM HRG or control vehicle (CN) for 1 to 5 minutes. Lysates were prepared and processed as described in Materials and Methods. In the upper panel, a sample of the total lysate was analysed by electrophoresis and then evaluated by immunoblot with antiphosphotyrosine antibody (IB:PY). In the lower panel, the lysate was first immunoprecipitated with antiphosphotyrosine antibody (IP:PY) before electrophoresis and Western blotting with anti-ER antibody (IB:ER). Human MCF-7 cell ER normally occurs as a 65- to 70-kd protein (Horigome et al., 1987). (**B**) MCF-7 HER-2 cells were treated in vitro with 10 nM HRG or control vehicle (CN) in the absence of estrogen to evaluate tyrosine phosphorylation of ER from 1 min to 4 hours using methods described in Materials and Methods. The total lysate was treated first by immunoprecipitation with anti-ER monoclonal antibody (IP:ER; not shown), followed by electrophoresis and immunoblotting with antiphosphotyrosine antibody (IB:PY)

and MCF-7 HRG cells (Figure 4B), and demonstrate that HRG-producing MCF-7 cells exhibit a specific estradiol binding capacity half that of control cells. Thus, either overexpression or activation of HER-2 elicits a down-regulation in ER similar to that found after treatment with estrogen.

It is also known that treatment of MCF-7 cells with estrogen leads to a pronounced down-regulation of ER transcripts. This process is believed to be mediated by an active ER and considered to be part of an autoregulatory circuit limiting the duration of estrogen action (Read et al., 1989; Ree et al., 1989; Borras et al., 1994). To test this phenomenon in our system, analyses of RNA from MCF-7 control and MCF-7 HER-2 cells was performed. These studies show that the major ER transcript of 6.5 kb is reduced in breast cells that overexpress HER-2 gene compared to controls (Fig. 5A), and that treatment of MCF-7 HER-2 cells in vivo with HRG elicits a similar decrease in ER transcripts over 72 hours (Fig. 5B).

Activation of the HER-2 Receptor Leads to Tyrosine Phosphorylation and Enhanced Nuclear Binding of the Estrogen Receptor

As noted above, phosphorylation of ER on tyrosine (Koffman et al., 1991; Migliaccio et al., 1991; Castoria et al., 1993) and serine (Arnold et al., 1994; Le Goff et al., 1994) residues has been associated with functional changes in hormone binding and nuclear localization and may represent a link to tyrosine kinase-mediated growth factor pathways. To test if ER is a substrate for phosphorylation by a tyrosine kinase receptor activated by HRG, we treated MCF-7 cells with HRG. MCF-7 control cells (which express normal amounts of the HER-2 receptor) treated with HRG in the absence of estrogen show a prominent increase in tyrosine phosphorylation of several cell proteins, especially at 185 kd, and demonstrate a marked time-dependent tyrosine phosphorylation of ER protein (Fig. 6A). Phosphorylation can be seen as early as 1 to 2 min after HRG treatment. In MCF-7 HER-2 cells, HRG promotes a similar acute increase in tyrosine phosphorylation of ER, with maximal phosphorylation occurring at 5-15 min and declining by 30 min (Fig. 6B). These results demonstrate a direct link between the HER-2/HRG pathway and ER tyrosine phosphorylation and are consistent with recent studies showing ligand-independent activation of steroid hormone receptors suggesting that molecular activation of ER may not depend exclusively on estrogen binding (Nelson et al., 1991; Ignar-Trowbridge et al., 1992; Smith et al., 1993).

To extend these observations to biologic phenomenon known to be associated with ER phosphorylation / activation, we performed a series of subcellular fractionation experiments in cells treated with HRG in the absence of estradiol. Published studies have shown that estrogen treatment rapidly enhances affinity of ER for

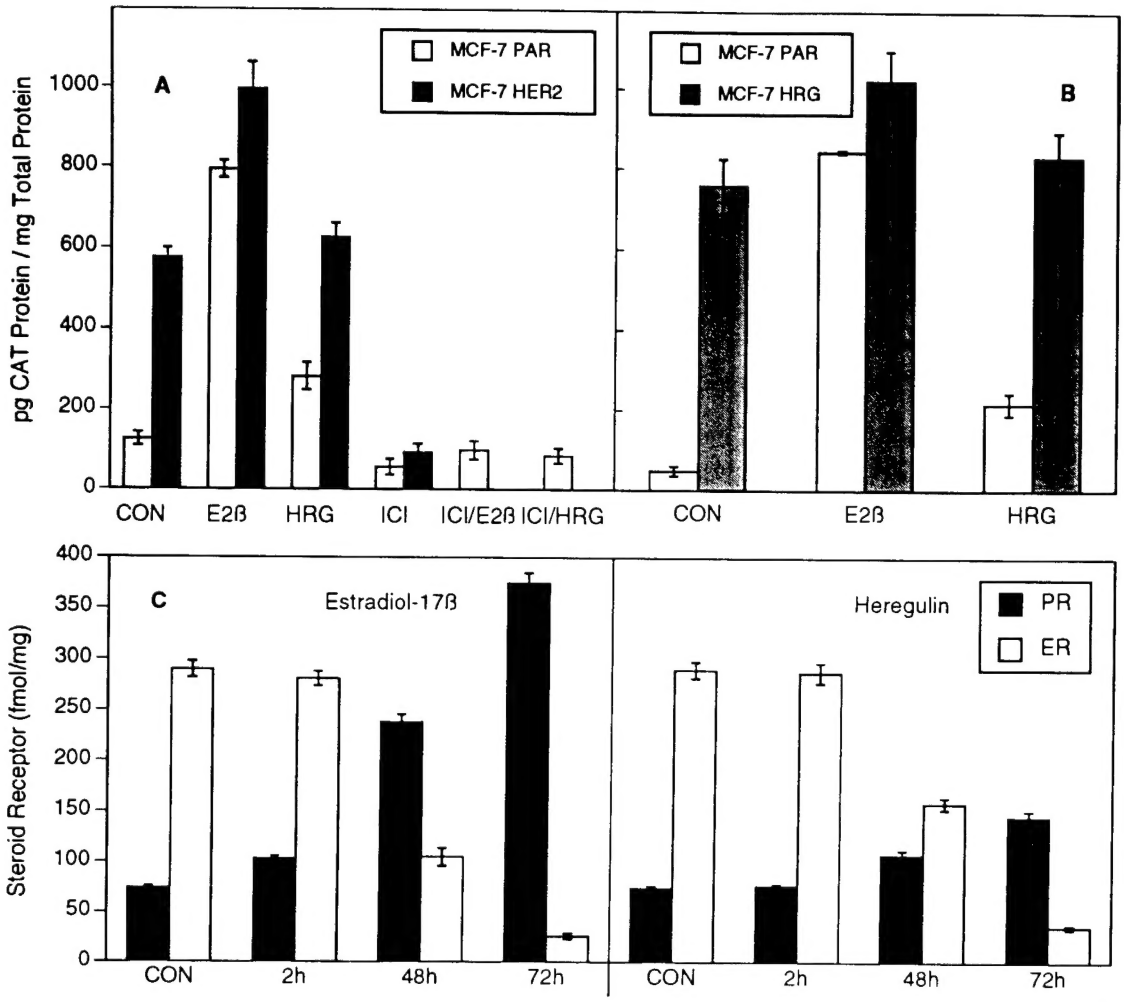

Figure 8 Activation of HER-2 receptor promotes estrogen-independent nuclear signaling by estrogen-responsive element (ERE) and production of estrogen-induced protein. (**A**) Heregulin promotes activation of ERE-CAT reporter gene after transient transfection in MCF-7 cells without (MCF-7 PAR) or with (MCF-7 HER2) overexpression of HER-2 gene. We have used a reporter plasmid containing a palindromic ERE, derived from the vitellogenin A2 promoter, and the CAT gene driven by a partial promoter sequence of thymidine kinase (Ernst et al., 1991; Lees et al., 1989). Substituting the basic reporter plasmid pBLCAT2 for pERE-BLCAT offers an additional control for specificity of the DNA-binding site in the regulatory sequence of the reporter gene (ERE). MCF-7 cells were used to establish transient transfection assays that allow the determination of ERE-dependent induction of CAT activity. CAT protein was assessed by established methods (De Maio & Buchman, 1990). Activity of control (CON), 1 nM estradiol-17ß (E2ß) and 10 nM heregulin (HRG) for 24h was assessed using these transfected cells with or without ERE. The specificity of this ligand-dependent CAT activation was verified by treatment of cells with the biologically inactive estradiol stereoisomer, estradiol-17α, and the non-estrogenic steroid, progesterone. At concentrations of 2 nM, both compounds have no significant effect on induction of CAT activity as compared to controls (data not shown). In addition, neither E2ß nor HRG elicited any change in the basal level of activity of the CON-CAT gene construct in MCF-7 parent cells. Further, both agents were ineffective in regulating the activity of ERE-CAT gene transiently transfected in HBL-100 breast cells which have no detectable level of endogenous ER (ER<5 fmol/mg protein; data not shown). The effect of preincubation with the pure antiestrogen, ICI 182,780 (ICI; 10 nM), on unstimulated MCF-7 HER2 cells and on the effects of estrogen and HRG in MCF-7 PAR cells was also tested. (**B**) Effect of 1 nM estradiol-17ß (E2ß) and 10 nM heregulin (HRG) on ERE-CAT reporter gene after transient transfection in MCF-7 cells without (MCF-7 PAR) or with (MCF-7 HRG) heregulin gene. Methods are described in Materials and Methods. (**C**) Timecourse of changes in estrogen receptor (ER) and progesterone receptor (PR), an estrogen-induced protein, in MCF-7 cells treated in vitro with 1 nM estradiol-17ß (left panel), 10 nM HRG (right panel) for 2h to 72h as compared to control solution treatments (CON). Specific steroid binding was determined using whole cell binding assays, and Scatchard analyses of the binding data were utilized to obtain quantitative estimates of the number of steroid binding sites/cell as before (Pietras & Szego, 1979). The results are shown as fmol specific hormone bound per mg cell protein

To further assess potential overlapping modes of action between HRG and ER pathways, the time course of changes in ER and progesterone receptor (PR), an estrogen-induced protein, were evaluated in MCF-7 cells treated in vitro with estrogen or HRG. Treatment of MCF-7 cells with estradiol-17ß for 2h to 72h results in a progressive induction of PR levels to more than 5-fold over control levels (P<0.001; Fig. 8C). Concurrently, estrogen elicits a complementary autoreduction in ER content to about 12% of that found in control cells (P<0.001). Treatment of MCF-7 cells with HRG over the same timecourse elicits a similar ER and PR phenotype in that PR binding capacity increases to twice that of control cells (P<0.001), while ER content falls to about 20 % of that found in control cells (Fig. 8C). Thus,

with ER in MCF-7 cells (Vignon et al., 1987; Read et al., 1989). Our findings demonstrate that heregulin supports growth of estrogen-dependent MCF-7 breast cells even in the absence of estrogen. Although independent work demonstrates that heregulins stimulate growth of breast cells with a complex of HER-2 and HER-3 receptors in vitro (Holmes et al., 1992; Carraway & Cantley,1994; Sliwkowski et al.,1994), neu differentiation factor, which is structurally homologous with heregulin, has been reported to promote differentiation and growth inhibition rather than growth (Bacus et al.,1992). Heregulin is known to bind with high affinity to heterodimers of HER-2 and HER-3 (Carraway & Cantley, 1994; Sliwkowski et al., 1994), and likely with complexes including HER-4 protein (Plowman et al.,1993; Dougall et al.,1994). Conflicting findings on the growth effects of heregulins (Staebler et al., 1994) may be attributable, in part, to different preparations of ligands or to differences in the cellular complement of class I receptors making up functional heterodimers in a given cell. The hypothesis that another member of the HRG ligand family elicits different effects on ER-positive and ER-negative cells has also been proposed by other investigators (Kung et al., 1994). Our data clearly demonstrate that, in estrogen-dependent MCF-7 breast cancer cells, HRG promotes and maintains cell growth even in the absence of estrogen. The inverse correlation between ER and HER-2 receptors in invasive cancer has been poorly understood (Adnane et al.,1989; Press et al.,1994), but the present data suggest that estrogen resistance due to long-term suppression of ER by HER-2-mediated pathways may require alternative therapeutic approaches.

Materials and methods

Cell Lines

MCF-7 cells (American Type Culture Collection, Rockville, MD) were stably transfected with a vector containing the full-length cDNA of human HER-2 gene from a primary breast cancer tissue (Slamon et al.,1987; Slamon et al.,1989b). These cells are termed MCF-7 HER-2. The vector used for introduction of HER-2 gene into human cells contained full-length human HER-2 gene ligated into a replication-defective retroviral expression vector, pLXSN (Slamon et al.,1987; Slamon et al.,1989b; Chazin et al.,1992). The latter was done by ligating a 3.8 kb Nco I to Mst II fragment with the full HER-2 coding sequence but lacking a polyadenylation signal into an amphotrophic retroviral expression vector containing a Moloney murine leukemia virus promoter, a neomycin phosphotransferase gene and a packaging signal, but devoid of viral protein coding sequences; thus rendering the virus replication-defective. Virus-producing cells were prepared by a transient rescue procedure as described elsewhere (Chazin et al.,1992). The vector devoid of HER-2 but containing neomycin phosphotransferase gene for selection (with G418) was packaged in an identical fashion and served as a control to infect MCF-7 cells (MCF-7 Control). G418-resistant clones were selected from MCF-7 cells infected with retroviral vector with or without HER-2 cDNA and assayed for expression of HER-2 receptor (Chazin et al.,1992). MCF-7 HER-2 cell clones with 2-5 copies of HER-2 gene per cell were used in these studies. Alternatively, in some experiments, pools of retrovirus-infected MCF-7 cells were selected first for HER-2 receptor overexpression by fluorescence-activated cell sorting using monoclonal anti-HER-2 receptor antibody 4D5 as described previously (Benz et al., 1992) and then screened further by subculture in the presence of G418.

Non-malignant human breast epithelial cells, HBL-100 (American Type Culture Collection), and all other cells were routinely plated in RPMI medium 1640 (GIBCO/BRL, Grand Island, NY) with 2mM glutamine and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA). Unless stated otherwise, medium with 10% heat-inactivated fetal bovine serum was used for standard plating conditions. In experiments requiring estrogen-free conditions, medium without phenol red and supplemented with 1% heat-inactivated, dextran-coated charcoal-treated fetal bovine serum was used for 72h prior to the start of the experiment. These are standard procedures for removing estrogens and steroid-like materials from culture media (Horigome et al.,1987; Welshons et al.,1993).

MCF-7 breast cancer cells with no endogenous production of HRG (American Type Culture Collection) were stably transfected with a vector containing full-length HRG-ß1 cDNA (Holmes et al.,1992). These cells are termed MCF-7 HRG. The vector used for introduction of HRG-ß1 gene into human cells contained full-length HRG-ß1 gene cloned into the expression vector pRK7 (Carter et al.,1992). This is a phagemid expression vector containing the human cytomegalovirus enhancer and promoter, a 5' intron and the simian virus 40 late polyadenylation signal. Expression vector devoid of HRG-ß1 cDNA was used as a control and was transfected into MCF-7 cells as described previously (Gorman et al., 1983). Production of HRG was assessed by competitive ligand-binding assay using [^{125}I]-HRG. With this assay, MCF-7 control cells showed no detectable level of HRG, while substantial levels were found in MCF-7 HRG cells (95% inhibition of ligand binding). A radioimmunoassay for HRG was also applied in selected experiments with use of a hamster monoclonal antibody to HRG (Holmes et al.,1992).

Heregulin-ß1 and Anti-HER-2 Antibodies

Heregulin-ß1 was prepared as described previously (Holmes et al., 1992; Sliwkowski et al.,1994). All preparations of recombinant HRG-ß1 were tested to verify high-affinity binding to HER-2/HER-3 heterodimers, and by phosphorylation of HER-2, as well as stimulation of proliferation of SKBR3 breast tumor cells (ATCC) in vitro (Holmes et al.,1992; Sliwkowski et al.,1994).

A humanized form of anti-HER-2 receptor monoclonal antibody 4D5 has been described before (Carter et al.,1992). The construct contains only the antigen-binding loops from murine 4D5 antibody in combination with human variable region framework residues plus IgG1 constant domains and is termed rhuMAb HER-2. Human IgG1 was used as control solution in appropriate experiments.

Quantitation of Cell Proliferation in Vitro

To assess proliferation of breast cells in vitro, aliquots of 4 x10^4 cells were plated in 96-well microdilution plates. For experiments with estradiol-17ß, cells were initially plated in estrogen-free media (see above). Following cell adherence, media supplemented with estradiol-17ß, estradiol-17α or

Nuclear) was determined using the same experimental approach. Binding data were analyzed by the method of Scatchard (Read et al.,1989; Welshons et al.,1993). In some experiments, cellular accumulation and binding of 4-hydroxy[N-methyl-^{3}H] tamoxifen (85 Ci/mmol; Amersham, Arlington Heights, IL) was assessed using methods reported elsewhere (Pietras & Szego,1979; Osborne & Fuqua,1994).

Determination of Estrogen Receptor Transcripts

In selected in vitro studies, cell RNA was isolated 7 days after plating MCF-7 control or MCF-7 HER-2 cells in estrogen-free media and subjected to Northern blot analysis (Slamon et al.,1987; Slamon et al.,1989a; Chazin et al., 1992), with hybridization of the resulting blot with a human ER cDNA (Green & Chambon,1988). In in vivo experiments using athymic mice, MCF-7 HER-2 cells were treated with exogenous HRG at 2 mg/kg sc every other day beginning with the time of cell inoculation. Paired mice were given vehicle control for comparison as described above. At selected times, tumor tissue was harvested and prepared for extraction of total RNA as previously described (Slamon et al.,1987; Slamon et al.,1989a). The resulting RNA was subjected to Northern blot analysis and hybridized with human ER cDNA.

Transient Transfection of Breast Cells with ERE-CAT Reporter Gene Constructs

A reporter plasmid containing a palindromic ERE and the chloramphenicol acetyltransferase (CAT) gene was used in these studies and is termed ERE-CAT (Lees et al., 1989; Ernst et al.,1991). In brief, an oligonucleotide sequence corresponding to an ERE derived from the vitellogenin A2 promoter of Xenopus leavis (-331 to -295) was cloned into the Xba I site of pBLCAT2. In addition, substitution of the basic reporter plasmid pBLCAT2 for pERE-BLCAT in selected experiments provided a control for specificity of the DNA-binding site in the regulatory sequence of the reporter gene (CON-ERE). MCF-7 cells used for transfections were cultivated in estrogen-free media for 5 days , then plated in 35-mm wells (3 x 10^5 cells / well) for 24 h. Prior to transfection, the cultures were washed three times in estrogen-free, serum-free media, and cells were then transfected with 2 µg DNA of the pERE-BLCAT plasmid using Lipofectamine® (GIBCO) as specified by the manufacturer. After incubation for 8h at 37° C, an equal volume of double-concentrated estrogen-free serum was added, and the medium was then replaced with estrogen-free medium at 24h. To determine ER-independent CAT activation, 2 µg per 35-mm dish pBLCAT2 was substituted for pERE-BLCAT. Activity of control vehicle, estradiol-17α (1 nM), estradiol-17ß (1 nM) and heregulin (10nM) was assessed using transfected cells with or without ERE-CAT. In some experiments, the pure antiestrogen, ICI 182,780 (7α-[9-(4,4,5,5,5-pentafluoropentysulfinyl) nonyl] estra-1,3,5(10)-triene-3,17ß-diol; generously provided by Zeneca Pharmaceuticals), was used to further assess the specificity of the assay system. Cells were harvested 24h later, and CAT protein was quantitated in cell extracts using a non-radioactive enzyme-linked immunosorbant assay (5 Prime-3 Prime, Boulder,CO) by established procedures (De Maio & Buchman,1990), with about 50 pg of CAT protein per ml of cell extract found to be the lower limit of detection. CAT reporter activity was normalized for the protein content in each sample.

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